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Isolation, characterization and identification of endophytic bacteria of *Hordeum vulgare* by molecular sequencing technique

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Abstract
Bacterial endophytes are ubiquitous colonizers of the inner plant tissues where they do not normally cause any substantial morphological changes and disease symptoms. In this paper we will give an overview of which bacterial species can live as endophytes, and how they enter a plant and live inside. We will also describe various bacterial traits which are required for a successful colonization of the plant's interior by endophytes. Some endophytes can promote plant growth and/or protect their host against phytopathogens. Many mechanisms of their beneficial action are predicted, but we will focus on those for which experimental support *in planta* was reported. Endophytic bacteria live symbiotically with the plant and in turn helping the plant in number of ways. The present investigations were undertaken to isolate and identify bacterial endophytes in roots of *Hordeum vulgare*. A total of two endophytic bacteria were isolated from the parts of the plant. On the basis of the morphological and biochemical characterization of the endophytes as well as 16S rRNA sequencing technique Studies on the optimization of growth of the isolates were performed by varying pH & temperature conditions. The ability of bacterial isolates was tested for different enzyme activity and also screened for sensitivity of different antibiotics.

Keywords: Endophytic bacteria, Biochemical characterization, *Hordeum vulgare*, 16S rRNA sequencing technique

Introduction
Barley (*Hordeum vulgare*) is a very important grain in the world today and it ranks the fourth in both quantities produced and in area of cultivation of cereal crops in the world. The annual world harvest of barley in the late century was approximately 140 million tons from about 55 million hectares. It is very versatile in every way and has been well adapted through its evolution. In fact, it is the most adaptable of the cereals. Much of the world's barley is produced outside of the regions where cereals such as maize and rice can grow well. It extends into the arctic or subarctic. Some species approach the subtropical Zone. Hordeum species are found in most areas with Mediterranean climate. The genus is also represented in zones with an oceanic as well as a continental climate (Rasmusson 1985). Barley also has a very good resistance to dry heat compared to other small grains. This feature allows it to grow near desert areas such as North Africa.

Barley is the fourth most cultivated cereal in the world, after wheat, maize and rice. The species is divided into three subgroups, six-row (*Hordeum vulgare*), two-row (*Hordeum distichum*) and intermediate (*Hordeum irregulare*), and both spring- and autumn-sown types are grown. The major use of barley is for animal feed, brewing malt and human food. Both two-row and six-row barley are used for malting, but the best malt quality for beer is produced from spring-sown two-row varieties. Barley is a short season, early ma-turing crop mainly adapted to a cool to temperate climate, but it can be found at the outer edges of agriculture, such as desert oases or the slopes of the Himalayas. Barley is not cultivated in the warm-humid climate of the tropics (Harlan, 1986).

Plant–microbe interactions that promote plant development and plant health have been the subject of considerable interest. Plants constitute vast and diverse niches for endophytic organisms. Among the microorganisms, endophytic bacteria occupy internal tissues of plants without causing damage to their host.

A broad range of agronomic and prairie plants common to the Bacterial endophytes i.e. bacteria that are present with in plants have been known for more than 120 years endophytic growth was recognized as a particular stage in the life of bacteria when it was described as an advance stage of infection and a close relation with mutualistic symbiosis. Endophytes have been defined as microorganism that could be isolated from surface sterilized plant organ

(Hennie and villforth,1940). Endophytic bacteria are referred to as those which can be detected at a particular moment within the tissues of apparently healthy plant hosts (Hallmann et al. 1997; Schulz and Boyle, 2006).

Most of the endophytes colonize different compartments of the plant apoplast, including the intercellular spaces of the cell walls and xylem vessels. Bacterial endophytes can be isolated from surface disinfected plant tissue or extracted from internal plant tissue. Both Gram positive and Gram negative bacterial endophytes have been isolated from several tissue types in numerous plant species. Furthermore, several different bacterial species have been isolated form a single plant (Kobayashi and Palumbo, 2000).

Endophytes enter plant tissue primarily through the root zone; however, aerial portions of plants such as flowers, stems and cotyledons, may also be used for entry. Specifically, the bacteria enter tissues via germinating radicals (Gagnc, *et al*, 1987) secondary roots, stomata's or as a result of foliar damage (Laban, *et al*, 1968) Endophytes inside a plant may either become localized at the point of entry or spread throughout the plant.

Materials and Methods

Collection of plant material

For the isolation of endophytic bacteria, healthy roots of Barley were collected from randomly selected healthy wild and cultivated plants from the Samples were placed in clean plastic bags, brought to the laboratory and used for further experimental purpose.

Isolation and purification of bacterial endophytes

The collected sample will be washed out with tap water to remove adhering soil, then they will be superficially sterilized with 70% ethanol and air dried under laminar air flow hood. The outer tissue will be removed from sample and inner tissue will be cut into small pieces and macerated in sterile pestle and mortar. Endophytic bacteria were isolated from roots. The plants were collected at the flowering stage. Roots were cut into sections 2.0-3.0 cm long. The tissue was put in beaker, soaked in distilled water and drained.

They were rinsed in 70% ethanol for 30 seconds and then sterilized for 4 minutes in sodium hypochlorite (3%) and then washed ten times with sterile water 12,10. Surface-disinfected tissue was aseptically macerated with homogenizers. Macerated tissue was diluted into 10-1 dilution by adding 9 volumes of sterile distilled water.

Serial dilution was made up to 10-6 dilution by taking 1 ml of well-shaken suspension and adding into 9 ml water blank tubes. 1 ml from appropriate dilutions were spread and plated on different media, Pikovskaya medium (PVK)28, yeast extract mannitol agar medium (YEMA)39, King's medium20, and tryptic soya agar (TSA)9, 4.

The plates were incubated at 28 °C for 3 days. The pure colonies were selected according to color and morphological characterization.

Pre-treatment

The roots of each plant were washed separately under tap water to remove adhering soil particles and the majority of microbial surface epiphytes is a part of pre-treatment.

Media for isolating endophytic bacteria

The choice of the growth medium is crucial as it directly affects the number and type of endophytic microorganisms that can be isolated from the root tissue. Nutrient agar media were used for the isolation of endophytic bacteria. Since there is no component in nutrient agar which can suppress the growth of endophytic fungi, so the media used for the isolation of endophytic bacteria were supplemented with an antifungal agent, nystatin at a concentration of 30 µg/mL of each to suppress fungal growth.

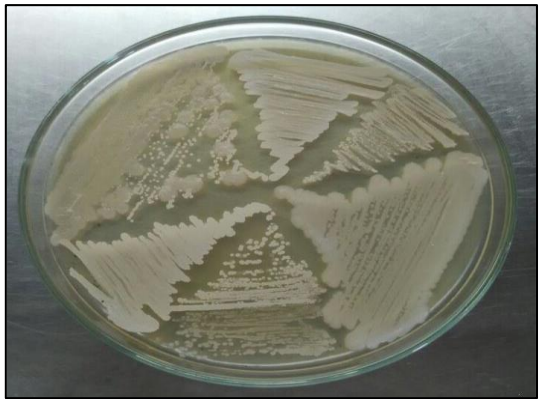

Fig 1: Diversity of endophytic bacteria on *pseudomonas* agar medium

Isolation, purification, and subculture of endophytic bacteria

After proper drying of surface sterilized plant material, using aseptic procedure the surface of the stems was removed using a sterile scalpel in the laminar air flow cabinet and roots were cut into pieces and each piece was placed on nutrient agar medium supplemented with antifungal agents. Plates with plant tissues are sealed using parafilm tape and incubated at 28±2 °C in order to recover the maximum possible colonies of bacterial endophytes. The observation was made for 48 hrs. After 24 hrs. from the bacterial cultures, morphologically different bacterial colonies were selected and are repeatedly streaked in order to achieve bacterial isolates. All selected isolates were subculture in nutrient agar slants and finally, all the purified endophytes were maintained at 4 °C till further used.

Physiological characterization

Physiological characterization based on temperature was done, to evaluate whether the endophytic bacteria showing thermostable nature. All the 40 isolates showing moderate growth at 28 °C, good growth 37 °C, and less growth at 48 °C. Optimum temperature for almost all the isolate was predicted 37 °C and no thermostable endophytic bacteria were detected. Another physiological characterization based on pH was done, isolates were showing optimum growth between pH range 6 to 8 and the growth of isolates abruptly declines at pH 9. Gupta et al., obtained similar results during the study of isolation of endophytic bacteria from *Prosopis cineraria* plant from root and leaf tissue. In physiological characterization they found the optimum growth of the isolates at pH 7 and optimum temperature 37 °C and thus confirm the findings.

Biochemical characterization

Table 1: Biochemical characterization of endophytic bacteria isolates of barley

Biochemical test		Entophytic bacteria	
		Positive (%)	Negative (%)
Gram's reaction		53.5	46.5
Oxidase		81.5	18.5
Catalase		100	-
Citrate utilization		16.3	83.7
Methyl red (MR)		67.5	32.5
Voges proskauer (VP)		7.0	93.0
Nitrate reduction (NR)		81.4	18.6
Carbohydrate Utilization Test	Different source of sugar	Positive (%) (acid production)	Positive (%) (gas production)
	Dextrose	21.0	2.3
	Mannitol	21.0	-
	Sucrose	27.9	5.0

Result and Discussion

Since the discovery of endophytes by Darnel, in 1904. Various investigators reported endophytic microbes from various plant exists in different ecosystems. It is not worthy that of the nearly 3,00,000 plant species that exists on earth each individual plant is host to one or more endophytes. Only a few these plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and interesting microorganism among myriads of plants in different settings and ecosystems is great.

Nutrient agar media has been used for the isolation of endophytic bacteria and different colonies have been recovered after culture plate. About 35 bacterial endophytes have been isolated in pure form from different plants used in this study. Purified culture of endophytic bacteria of *H. vulgare* is shown in Fig. From the total 35 isolates, 10 isolates were selected for further investigation.

These studies demonstrated the occurrence and diversity of culturable endophytes. In India, only countable number of the reports showed the diversity of endophytic bacteria and fungi from the barley plants. There is no report on endophytic bacteria from Bihar. There are reports on the isolation of fungal endophytes from *C. roseus*, *O. sanctum*, *M. arvensis*, and *S. rebaudiana*, but there are only few reports on the isolation of endophytic bacteria. In general, endophytic bacteria occur as lower population density than rhizosphere bacteria or bacterial pathogen. The surface of plants carries a wide range of microbial contaminants. To avoid this source of infection and for the isolation of endophytes, explants must be thoroughly surface-sterilized before inoculating them onto the nutrient medium.

Conclusion

The study of entophytic bacteria is a challenging field of research, from a fundamental as well as an applied focus. The first attempts to use endophytic bacteria for the improvement of the plant. *H. vulgare* have been promising, but considerable research efforts are required to optimize the practical applications. More knowledge of the population dynamics and activity of endophytic bacteria in their host plants is required.

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